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TITLE: Role of the Adherens Junction Protein Fascin in the Regulation of Tight Junction Permeability in the Mouse Mammary Gland

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

An adenovirus based gene delivery system was developed which allows the delivery of target genes to the mouse mammary gland epithelium and to cultured mouse mammary epithelial cells. Transduction of the mammary gland does not induce inflammation or effect tight junction permeability. Transduced cells are morphologically normal and produce milk. This gene delivery system was used to express an N-terminally truncated mutant of the tight junction protein occludin in the mammary gland and in cultured cells. Transgene expression caused programmed epithelial cell death (anoikis) in the mammary gland and in cultured cells. Truncated occludin localized to the tight junction 18 hours post transduction and showed vesicular distribution 48 hours post transduction. The tight junction protein ZO-1 left the tight junction and colocalized with the transgene. The apical junction complex, actin bundling protein fascin was up-regulated and diffusely distributed in transduced cells. The fascin binding, adherens junction protein β-catenin was distributed to the perinuclear region of transduced cells.

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Introduction:

The tight junction is an array of anastomosing proteinaceous fibers lying in the apicolateral membrane regions of adjacent epithelial cells. These fibers lie in parallel along their lengths and are thought to interact via hetero and homophilic interactions among the occludin and claudin proteins composing these fibers. The C termini of occludin and the claudins have been shown to interact with membrane associated guanylate kinases (MAGUKs), such as ZO-1, the first tight junction protein to be cloned. Tight junction associated MAGUKs and other proteins form an intercellular plaque that runs continuously along the tight junction fibrils. This plaque connects the occludin and claudins in tight junction fibrils to actin stress fibers, which in turn connect to the perijunctional actin ring. The tight junction complex lies at the apical boundary of the cadherin based adherens junction complex. The tight junction, the adherens junction, and the perijunctional actin ring comprise the apical junctional complex. The cadherin based adherens junction occurs basal to the tight junction and, like the tight junction, runs continuously along the apicolateral border of adjacent epithelial cells. The mammary adherens junction is largely comprised of the transmembrane protein E-cadherin that forms homophilic interactions between adjacent cells. An intercellular plaque runs along the intercellular C-termini of the cadherin molecules. This plaque is comprised of α-catenin (vinculin), β-catenin, and γ-catenin (plakoglobin). The catenin based plaque connects the transmembrane cadherins to actin stress fibers that in turn connect to the perijunctional actin ring. The architecture of the actin stress fibers and the perijunctional actin ring itself is dependent upon the actin bundling protein fascin (see figure 1).

The architecture of the apical junction complex is almost always perturbed in breast cancer. ZO-1, E-cadherin, all of the catenins, and fascin are very frequently irregularly expressed in breast cancer. The perijunctional actin ring is usually poorly defined in breast cancer. Up-regulation of fascin is a feature of transformed cells in general.

A great deal of scientific research has been devoted to intracellular signaling downstream of cadherin binding events and it has been shown that proper cadherin mediated cell/cell contact is necessary for normal differentiated epithelial cell survival. I hypothesize that there is also intracellular signaling from the tight junction and that proper occludin mediated cell/cell contact is necessary for mammary epithelial cell survival. The cells comprising an aggressive breast tumor are by definition able to survive at least some degree of improper cell/cell binding. Understanding the survival dependency of healthy epithelial cells upon proper cell/cell contact will be of great value in fighting breast cancer.

This project is currently testing the hypothesis that mammary epithelial cells require normal occludin mediated tight junction cell/cell attachment for survival. The roles played by the actin bundling protein fascin and the fascin binding, adherens junction protein β-catenin during programmed cell death are being studied.

Body:

1. A gene delivery system to the mouse mammary epithelium and to cultured mouse mammary epithelial cells was established based on an E1/E3 deleted adenovirus expression construct which encodes the GFP reporter gene. It was shown that this construct could be used via intraductal injection of the mouse mammary gland to transduce the mouse mammary epithelium. After trying various viral dosages for various lengths of transduction, it was established that 10^7 plaque forming units (pfu) of E1/E3 deleted adenovirus expressing either the green flourescent protein (GFP) reporter or the LacZ reporter could be used effectively to transduce the fourth mammary gland of CD-1 mice during late pregnancy. Transduction did not cause inflammation or result in the increase of tight junction permeability which is a hallmark of mastitis (figure 2). Transduction did not effect the switch from pregnancy to lactation or the concurrent decrease in mammary epithelial tight junction permeability. Lipopolysaccharide (LPS), a cell wall component of gram negative bacteria known to induce mastitis, was injected intraductally into the mammary gland to verify the ability of the ^{14}C-sucrose assay to detect mastitis. "TP" in the legend of figure 2 refers to adenoviral terminal protein deletion. The TP deleted adenovirus showed no advantages over the E1/E3 deleted construct and was dropped from the study. Transduced cells during day 2 of lactation were shown via fluorescent microscopy to secrete milk protein and milk fat globules normally (figure 3). **CONCLUSION:** E1/E3 deleted adenovirus expression constructs can be used effectively in the mouse mammary gland to drive transgene expression without deleterious effects.

2. To test whether the adenovirus technique can be used to examine tight junction function and affect the distribution of apical junction complex proteins, an E1/E3 deleted adenovirus was created which encodes an N-terminally truncated occludin mutant comprised of the last 14 amino acids of the second extracellular loop of occludin, the second transmembrane domain of occludin, and the occludin cytoplasmic tail. This virus also expresses the GFP reporter gene from a separate promoter as an entirely separate and cytoplasmic protein (figure 4). The truncated occludin was expected to disrupt the tight junction and was expressed via adenoviral transduction in mammary epithelial cells grown on filters. Transgene expression appeared to cause disruption of the tight junction specifically and the apical junction complex as a whole. The transgene localized to the tight junction at 18 hours post transduction but showed vesicular distribution at 48 hours. The tight junction protein ZO-1 colocalized strongly with the transgene at 18 and 48 hours post transduction (figures 5 and 6). **CONCLUSION:** E1/E3 deleted adenovirus can be used to deliver transgenes to the apical junction complex in mouse mammary epithelial cells and effect apical junction complex properties.

3. The loss of normal apical junction complex structure resulted in epithelial programmed cell death (anoikis). The truncated occludin transgene was expressed in cultured mouse mammary epithelial cells and in the mouse mammary gland via adenovirus transduction. Cells expressing the transgene were shedding into the lumen of the mammary gland at 18 hours post transduction. Alveoli in which the majority of cells were transduced were highly disorganized at 18 hours. Transduced cells were largely cleared from the mouse mammary epithelium at 72 hours post transduction (figure 7). The TUNEL assay for apoptosis was performed on filter grown mouse mammary epithelial cells. At 48 hours post transduction there were a great number of TUNEL+ nuclei in these cultures (figure 8). **CONCLUSION:** The disruption of the apical junction complex induced by the expression of N-terminally truncated occludin causes programmed cell death in the mouse mammary gland epithelium and in a non-transformed mouse mammary epithelial cell line.

4. Fascin is distributed mainly at the perijunctional actin ring in normal mammary epithelium. Non transduced mammary epithelial cells were processed for immunofluorescence using the Dako mouse monoclonal antibody against fascin (figure 9 inset G).

5. Expression of N-terminally truncated occludin in cultured mammary epithelial cells resulted in the redistribution of fascin from the perijunctional actin ring to the cytoplasm. The truncated occludin transgene was expressed in cultured mouse mammary epithelial cells via adenovirus transduction. At 28 hours post transduction the actin staining in transduced cells was punctate, diffusely perijunctional, and significantly more

intense than in surrounding non-transduced cells suggesting that fascin becomes up-regulated and/or more accessible to the antibody in response to transgene expression (figure 9). Note that inset G in figure 9 shows fascin staining in non-transduced cells and that this image was exposed for a longer time than the images showing fascin distribution in 28 hour transduced cells. At 48 hours post transduction fascin staining in transduced cells was truly cytoplasmic as demonstrated by its colocalization with the cytoplasmic GFP reporter. Fascin staining was much brighter in transduced cells than in surrounding non-transduced cells . Note that the transduced cells shown in figure 10 are apoptotic and have ejected their nuclei. **CONCLUSION:** The disruption of the apical junction complex induced by the expression of N-terminally truncated occludin causes a redistribution of the actin bundling protein fascin from the perijunctional actin ring to the cytoplasm.

6. Expression of N-terminally truncated occludin in cultured mammary epithelial cells resulted in the redistribution of the adherens junction protein β-catenin to the perinuclear region. The truncated occludin transgene was expressed in cultured mouse mammary epithelial cells via adenovirus transduction. β-catenin partially colocalized with the transgene at 48 hours (figure 11). The monolayer in figure 11 is not perfectly flat and the transduced cells are leaving the monolayer; nevertheless the normal localization of β-catenin to the adherens junction can be seen in those of the neighboring non-transduced cells which are in the same focal plane as the transduced cells. It has been demonstrated elsewhere that β-catenin binds fascin in a non-cadherin complex and localizes with fascin at the leading edge of migrating cells. The perinuclear localization of β-catenin during anoikis is well established. **CONCLUSION:** The disruption of the apical junction complex induced by the expression of N-terminally truncated occludin causes a redistribution of the fascin binding, adherens junction protein β-catenin to the perinuclear region.

List of Key Research Results:

- I have confirmed the feasibility of fascin immunofluorescence in a mammary epithelial cell line pertaining to task 1. I am in the process of completeing sub-parts a&b.
- I have demonstrated my ability to construct adenovirus vectors and use them in the transduction of the mouse mammary gland epithelium pertaining to task 2. I am in the process of constructing a fascin expressing adenovirus.
- I have demonstrate my ability to use adenovirus transduction of the mouse mammary gland epithelium to study tight junction dynamics pertaining to task 3.
- I have demonstrated dynamic redistribution of three apical junction proteins (ZO-1, fascin, and β-catenin) in response to perturbation of the tight junction pertaining to task4.

List of Reportable Outcomes:

- Poster presentation at DOD Era of Hope 2000 in Atlanta
- Poster presentation at Gordon Research Conference for Mammary Gland Biology 2001 in Bristol N.H.
- December 2001 poster presentation at the American Society for Cell Biology in Washington D.C. (abstract enclosed).

Conclusions:

An adenovirus based gene delivery system was developed which allows the delivery of target genes to the mouse mammary gland epithelium and to cultured mouse mammary epithelial cells. Transduction of the mammary gland does not induce inflammation or effect tight junction permeability. Transduced cells are morphologically normal and produce milk. This gene delivery system was used to express an N-terminally truncated mutant of the tight junction protein occludin in the mammary gland and in cultured cells. Transgene expression caused programmed epithelial cell death (anoikis) in the mammary gland and in cultured cells. Truncated occludin localized to the tight junction 18 hours post transduction and showed vesicular distribution 48 hours post transduction. The tight junction protein ZO-1 left the tight junction and colocalized with the transgene. The apical junction complex, actin bundling protein fascin was up-regulated and diffusely distributed in transduced cells. The fascin binding, adherens junction protein β-catenin was distributed to the perinuclear region of transduced cells.

Abstract accepted by the American Society for Cell Biology for poster presentation in Washington D.C. December 2001

The expression of N-terminally truncated occludin causes programmed cell death in the mouse mammary gland epithelium and in a non-transformed mouse mammary epithelial cell line.

In order to study the effect of tight junction disruption in the mammary epithelium, an E1/E3 deleted adenovirus expression vector was used to express an N-terminally truncated, N-terminally FLAG tagged mutant of the tight junction protein occludin in the mouse mammary epithelium and in cultured mouse mammary epithelial cells. This construct and other similar occludin truncation constructs have been shown to perturb tight junction function in non-mammary transformed cell lines. An adenovirus encoding both truncated occludin and the green fluorescent protein (GFP) reporter gene was injected intraductally into the mammary glands of female mice on day 19 of pregnancy. Transduced cells had pycnotic nuclei and began to shed into the mammary lumen 18 hours after injection. They were almost entirely cleared from the mammary gland three days following injection. Epithelial cells in control glands, injected with an adenovirus construct that expresses only GFP, appeared normal and secreted milk protein and milk fat. To examine induction of programmed cell death in more detail, filter grown immortalized non-transformed mouse mammary epithelial CIT3 cells were transduced with the same adenovirus constructs. In cells expressing truncated occludin, the tight junction protein ZO-1 moved to the cytoplasm and the adherens junction protein beta-catenin showed a punctate, perinuclear distribution. Subsequently, cells expressing truncated occludin ejected their nuclei, underwent apoptotic blebbing, and detached from the monolayer. Many nuclei were TUNEL positive in these cells. Control cells appeared normal. We conclude that the expression of truncated occluding causes programmed cell death in the mouse mammary gland epithelium and in the CIT3 mouse mammary epithelial cell line. Supported by DOD grants BC971759 and BC 9900.

Figure 1

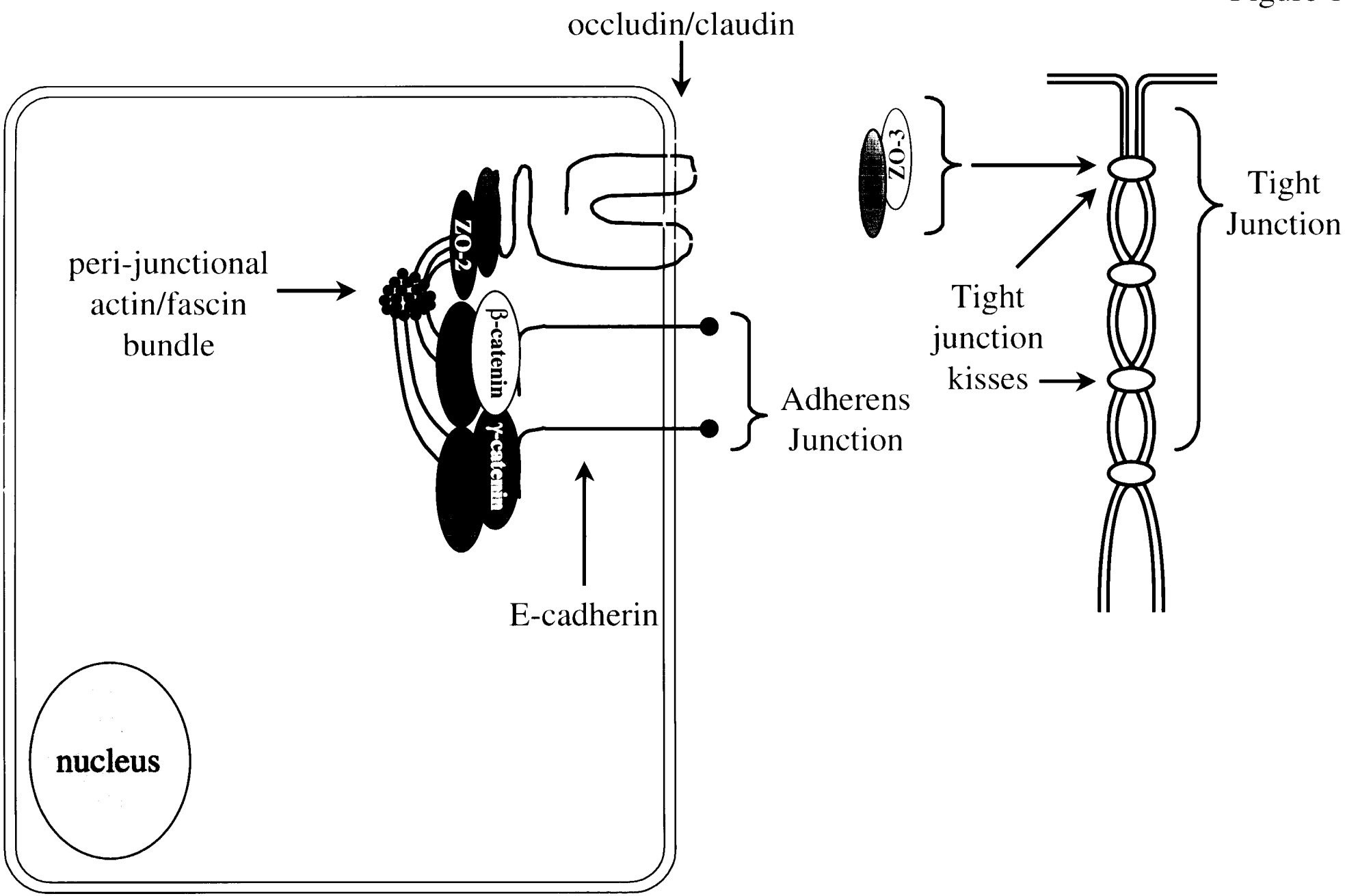

Figure 1. Diagramatic sketch of the apical junction comlex of an epithelial cell:

Figure 2

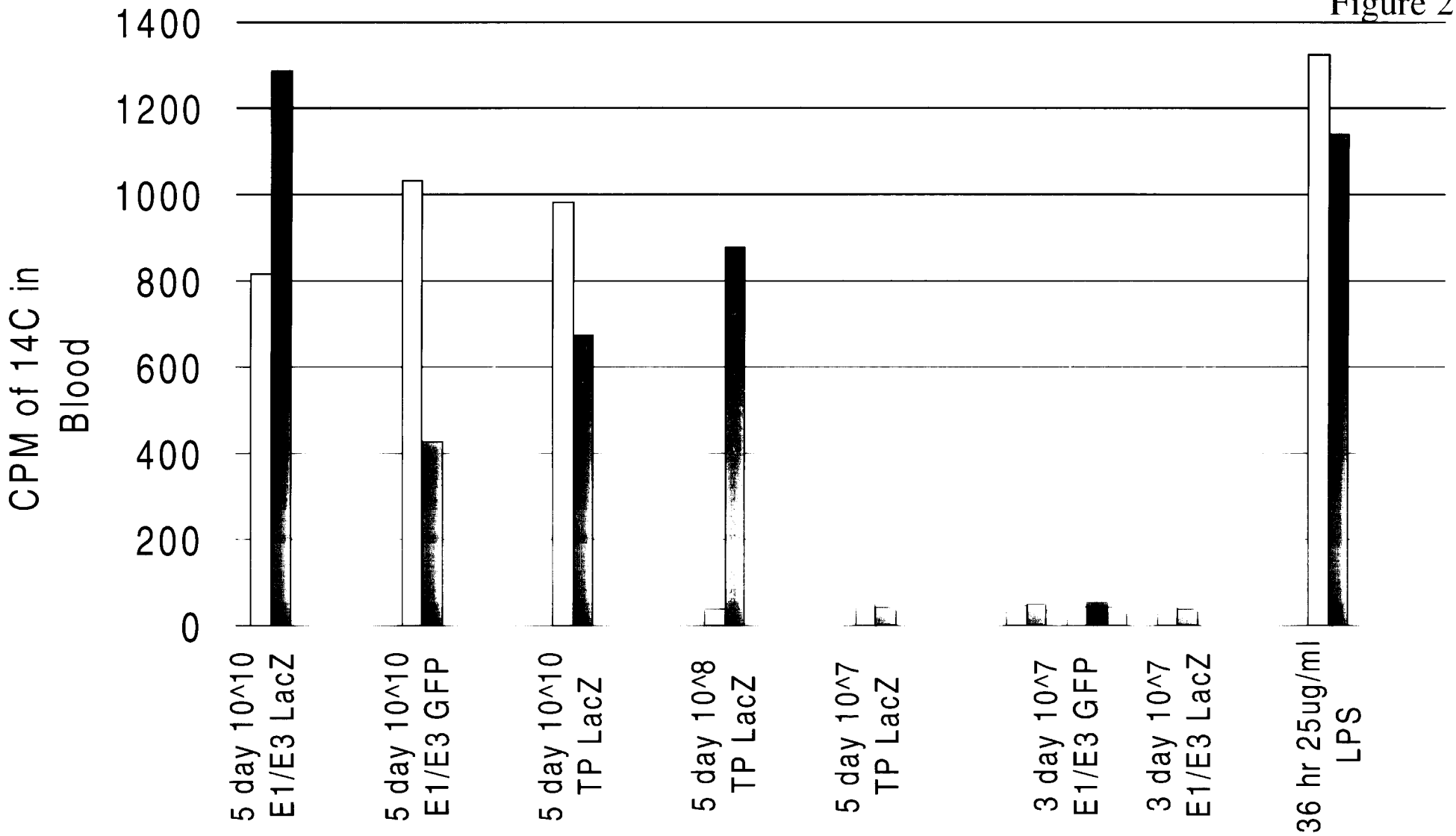

Figure 2. The effect of viral dosage and length of transduction on mammary gland permeability:

Mice were intra-ductally injected into the fourth mammary gland on the 19th day of pregnancy with various amounts of adenovirus and sacrificed at various time points following virus injection. The 14C sucrose permeability assay was then performed as follows: for each mouse, 14C sucrose was first injected into the non-virally treated contra-lateral gland and a tail vein blood sample was collected 5 minutes post sucrose injection. 14C sucrose was then injected into the virally transduced gland and a second tail vein blood sample was taken. The blood 14C sucrose level was obtained via liquid scintillation counting and the 14C sucrose level obtained after the first injection was subtracted from the level obtained after the second injection. Each colored bar represents the blood 14C sucrose level of one mouse and represents the permeability of the virally transduced mammary epithelium to 14C sucrose.

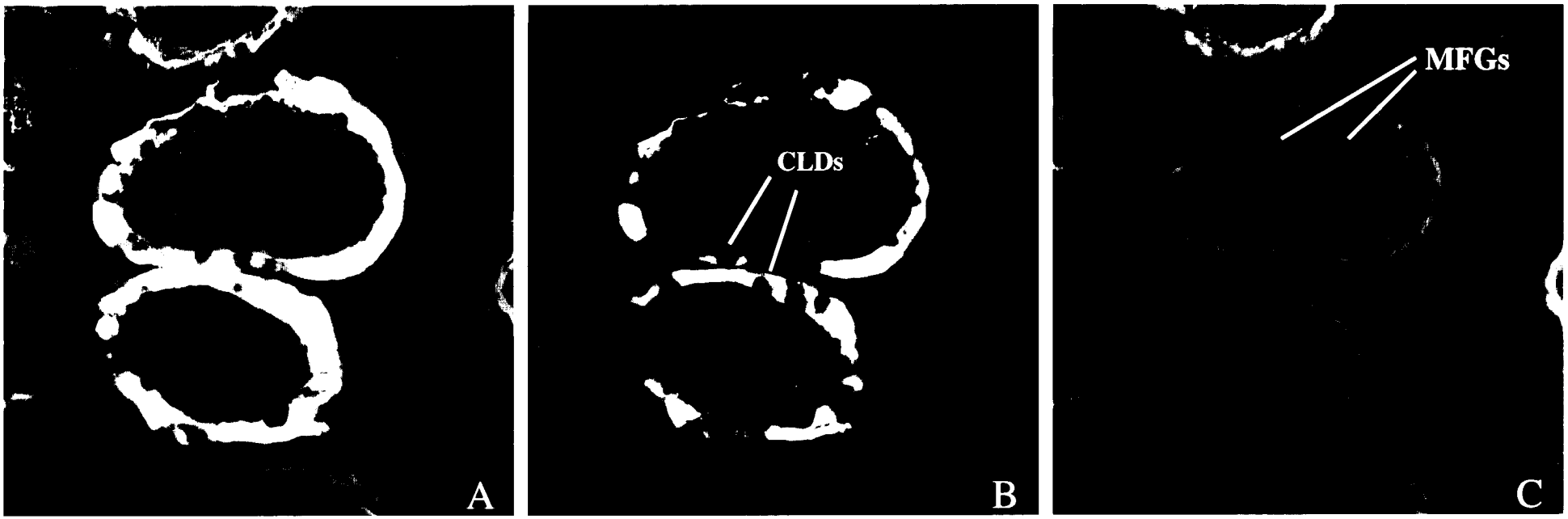

Figure 3. Adenovirus transduction of the mammary gland epithelium:

A female mouse was injected intraductally into the fourth mammary gland with 10^7pfu of an E1/E3 deleted adenovirus expression vector encoding GFP on day 19 of pregnancy. The mouse was sacrificed three days post injection and the virally injected mammary gland was processed for frozen sectioning. Frozen sections were obtained and stained with dapi nuclear dye (blue) and Cy3 conjugated wheat germ agglutinin (red). Wheat germ agglutinin stains the apical membrane of cells and also stains the membrane surrounding released milk fat globules (MFGs) but does not bind to cytoplasmic lipid droplets (CLDs) within the cell. Panels A-C show two adjacent alveoli which are highly transduced: A-C 20x; A = dapi, Cy3 wheat germ aglutinin, and GFP; B = GFP; C = Cy3 wheat germ aglutinin.

Figure 4

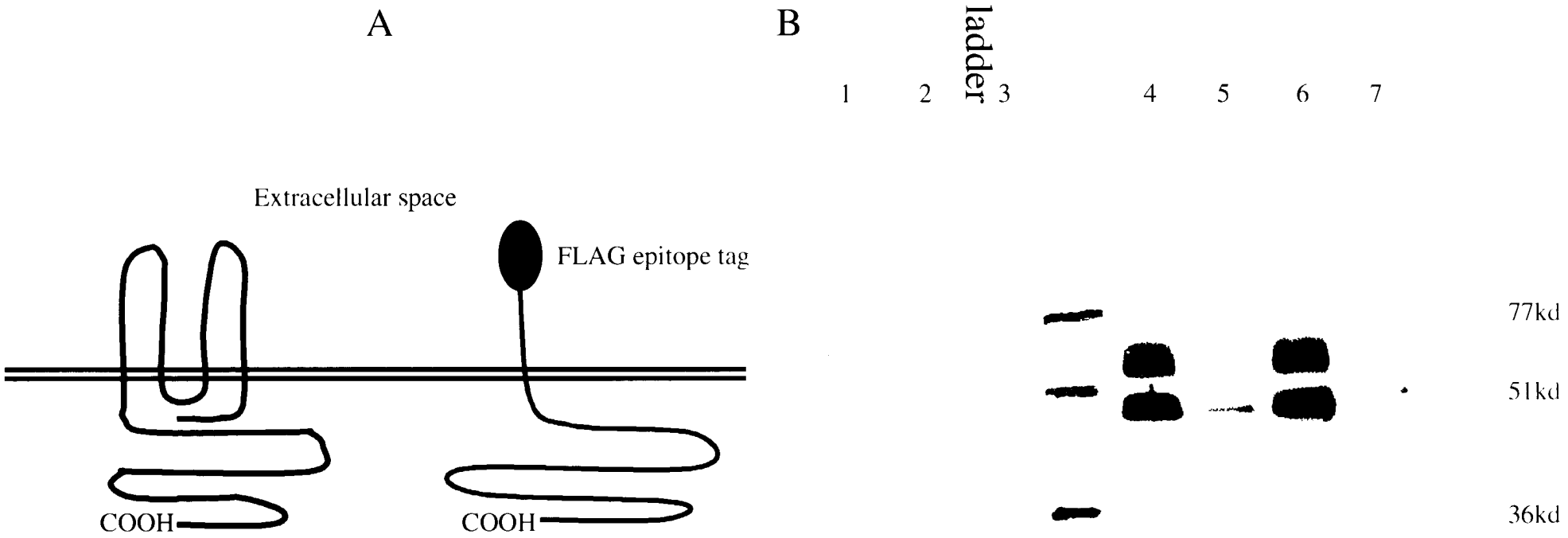

Figure 4. An E1/E3 deleted adenovirus expression construct was created to drive the expression of the the FLAG tagged N-terminally truncated occludin construct in transduced cells:
A: Diagramatic sketch of the topography of wild type occludin on the left and the FLAG tagged N-terminally truncated occludin construct on the right as it would appear at the apicolateral membrane of a target cell.
B: Western blot of whole cell lysate from adenovirus transduced cells: lanes 1 and 2 are serial dilutions of non-infected 293 cells. Lane 3 is a known positive sample from Eph-4 mammary epithelial cells which express occludin. Lanes 4 and 5 are serial dilutions of 293 cells infected with a plaque purified clone, and 6 and 7 are serial dilutions of 293 cells infected with a different plaque purified clone. The lower running doublet in lanes 4 through 7 represents the N-terminally truncated occludin construct.

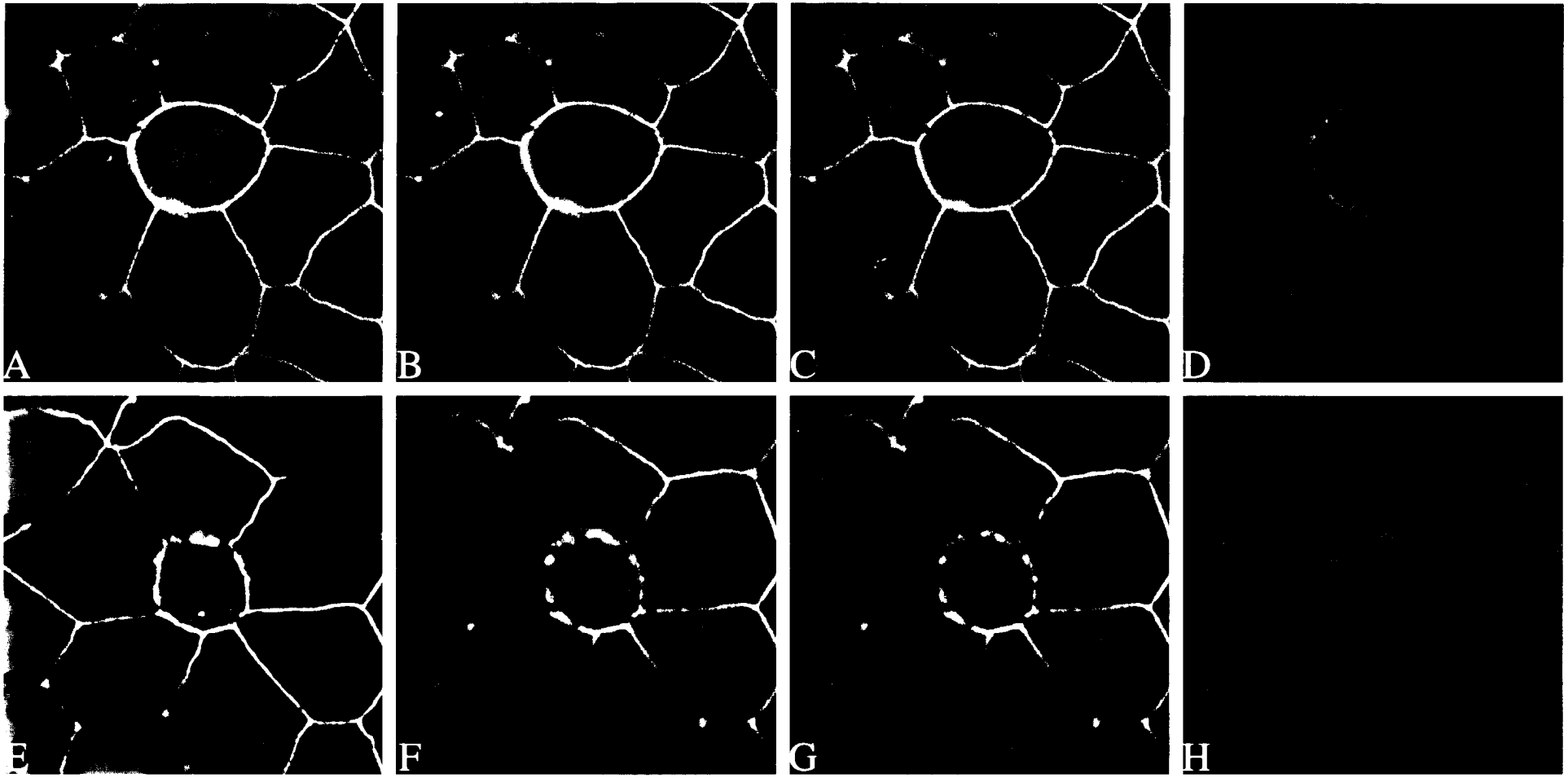

Figure 5. The FLAG tagged N-terminally truncated occludin construct localized mainly at the tight junction 18 hours post transduction:

Filter grown CIT3 cells were treated with a multiplicity of infection (moi) of ~50pfu/cell of an E1/E3 deleted adenovirus expression vector encoding the occludin transgene and GFP. The filters were processed for immunofluorescence 18 hours following viral treatment. Filters were incubated with a mouse monoclonal antibody against FLAG and a rat monoclonal antibody against ZO-1. Sections were then treated with Cy3 (red) conjugated anti-mouse and Cy5 (shown in green) conjugated anti-rat secondary antibodies: A-H = 100x; A and E = FLAG, dapi, and ZO-1; B-D are same cell as A; F-H are same cell as E; B and F = FLAG and ZO-1; C and G = ZO-1; D and H = FLAG; F-H are 0.5µm basal to the plane of the tight junction as in E

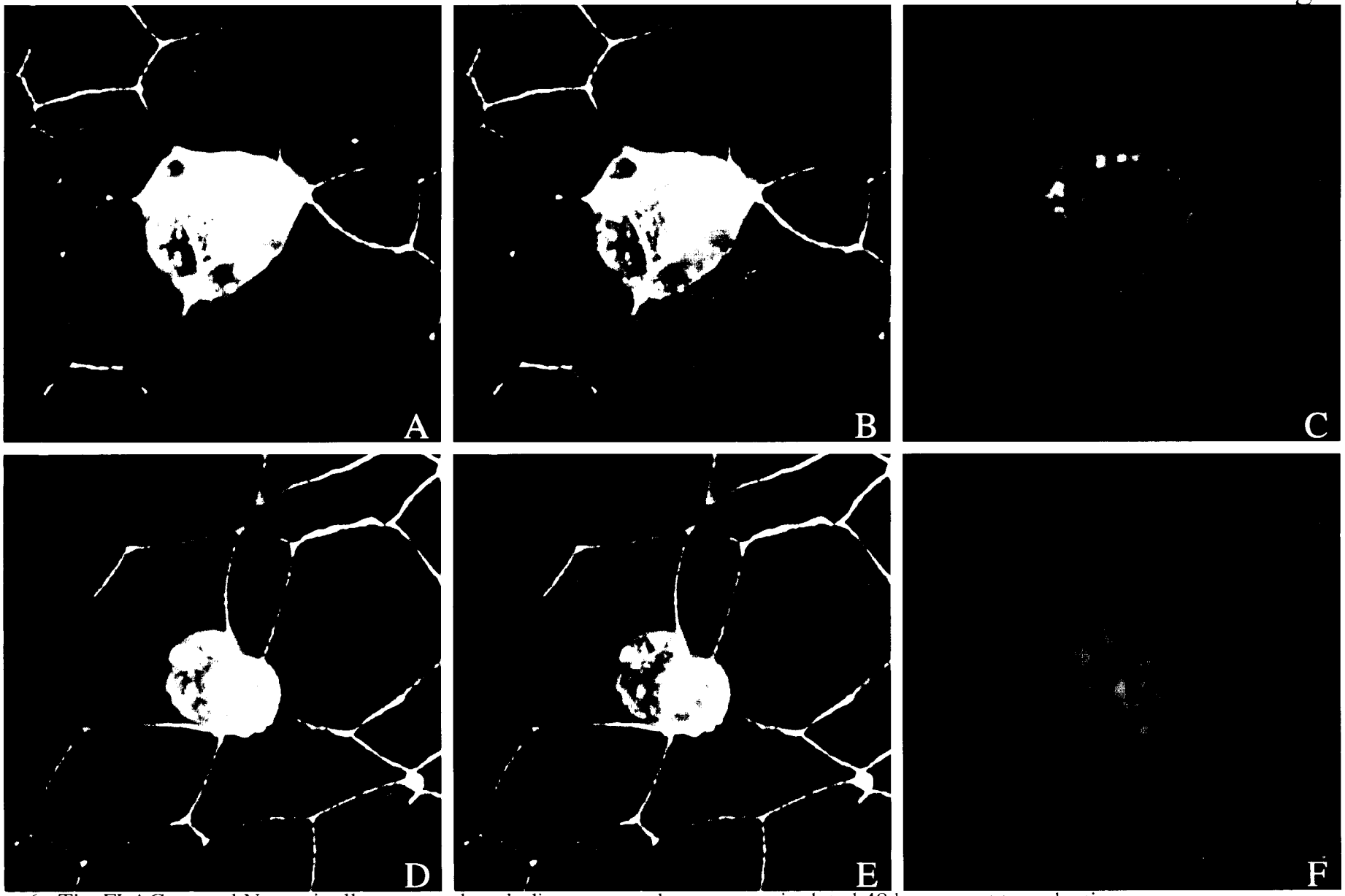

Figure 6. The FLAG tagged N-terminally truncated occludin construct became vessiculated 48 hours post transduction:

Filter grown CIT3 cells were treated with an moi of ~50pfu/cell of an E1/E3 deleted adenovirus expression vector encoding the occludin transgene and GFP. The filters were processed for immunofluorescence 48 hours following viral treatment. Filters were incubated with a mouse monoclonal antibody against FLAG and a rat monoclonal antibody against ZO-1. Sections were then treated with Cy3 (red) conjugated anti-mouse and Cy5 (shown in green) conjugated anti-rat secondary antibodies: A-F = 100x; A-C = same cell; D-F = same cell; A and D = FLAG and ZO-1; B and E = ZO-1; C and F = FLAG

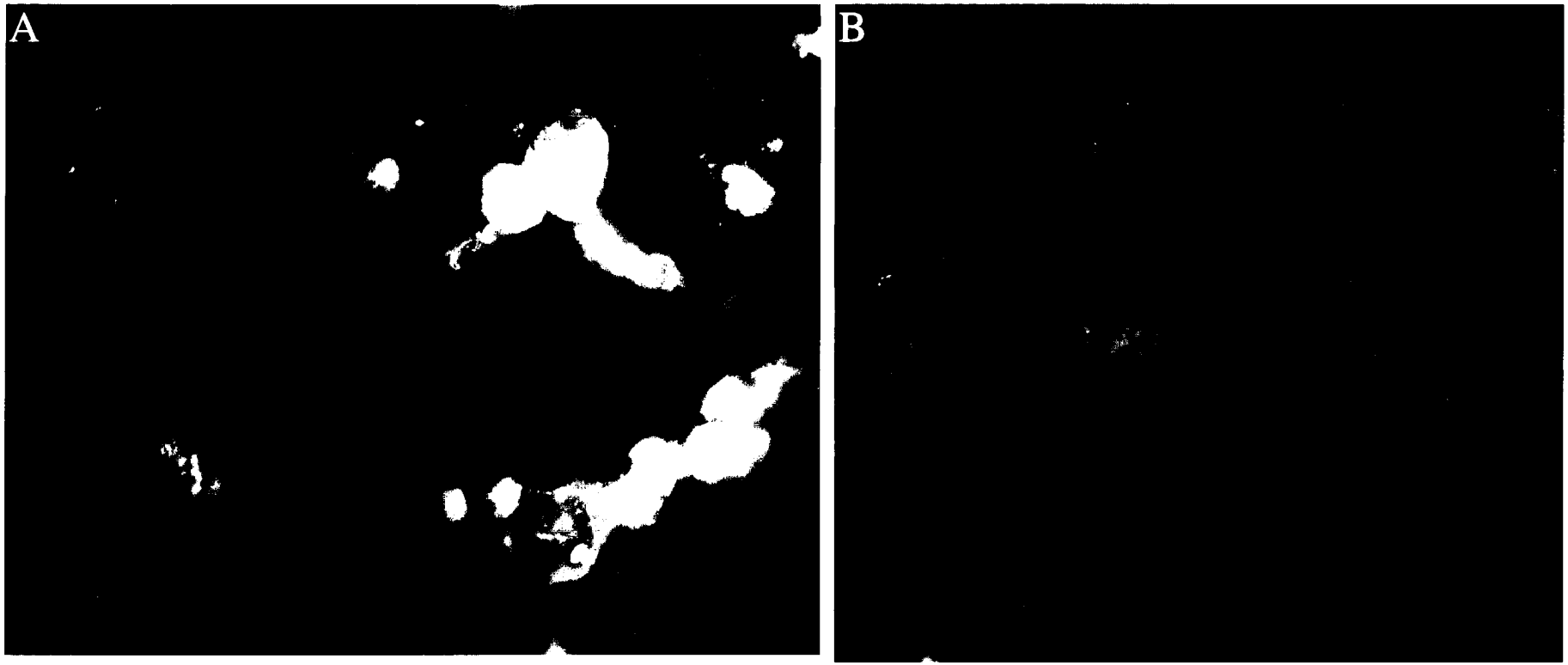

Figure 7. Virally transduced cells were cleared from the mammary gland epithelium at 72 hours:

Two female mice were injected intraductally into the fourth mammary gland with 10^7pfu of an E1/E3 deleted adenovirus expression vector encoding the FLAG tagged N-terminally truncated occludin construct and GFP on day 19 of pregnancy. One mouse was sacrificed 18 hours post injection and the other was sacrificed 72 hours post injection. The virus injected mammary glands were processed for frozen sectioning. Sections were incubated with a mouse monoclonal antibody against FLAG and then treated with Cy3 (red) conjugated anti-mouse secondary. Antibody treated sections were then stained with dapi nuclear stain (blue): A and B = 100x; A = FLAG, dapi, and GFP at 18 hours post injection; B = FLAG and dapi at 72 hours post injection.

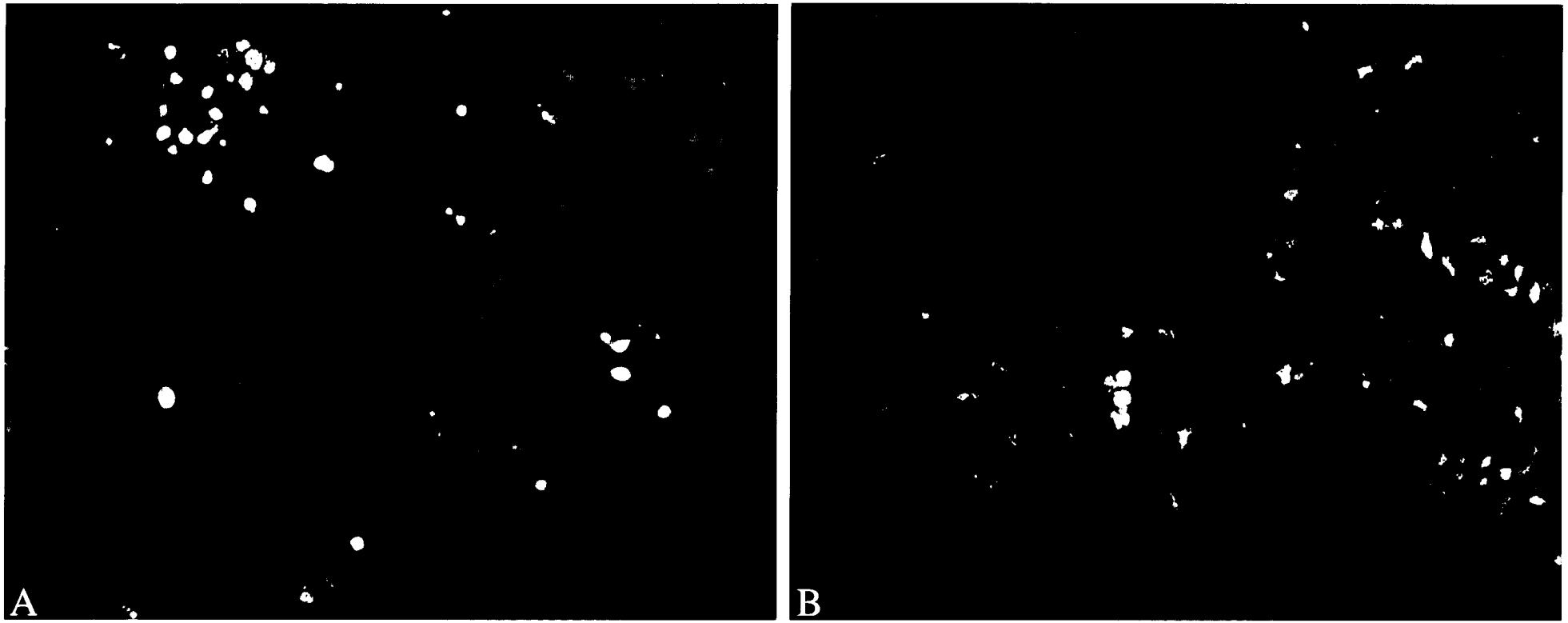

Figure 8. Expression of the truncated occludin construct caused apoptosis :

Filter grown CIT3 cells were treated with an moi of ~50pfu/cell of an E1/E3 deleted adenovirus expression vector encoding the FLAG tagged N-terminally truncated occludin construct and GFP (A) or ~50pfu/cell of an E1/E3 deleted adenovirus expression vector encoding GFP only (B). The filters were processed for TUNEL staining 48 hours following viral treatment: A and B 20x ; TUNEL positive nuclei are red; GFP positive cells and cellular debris are green.

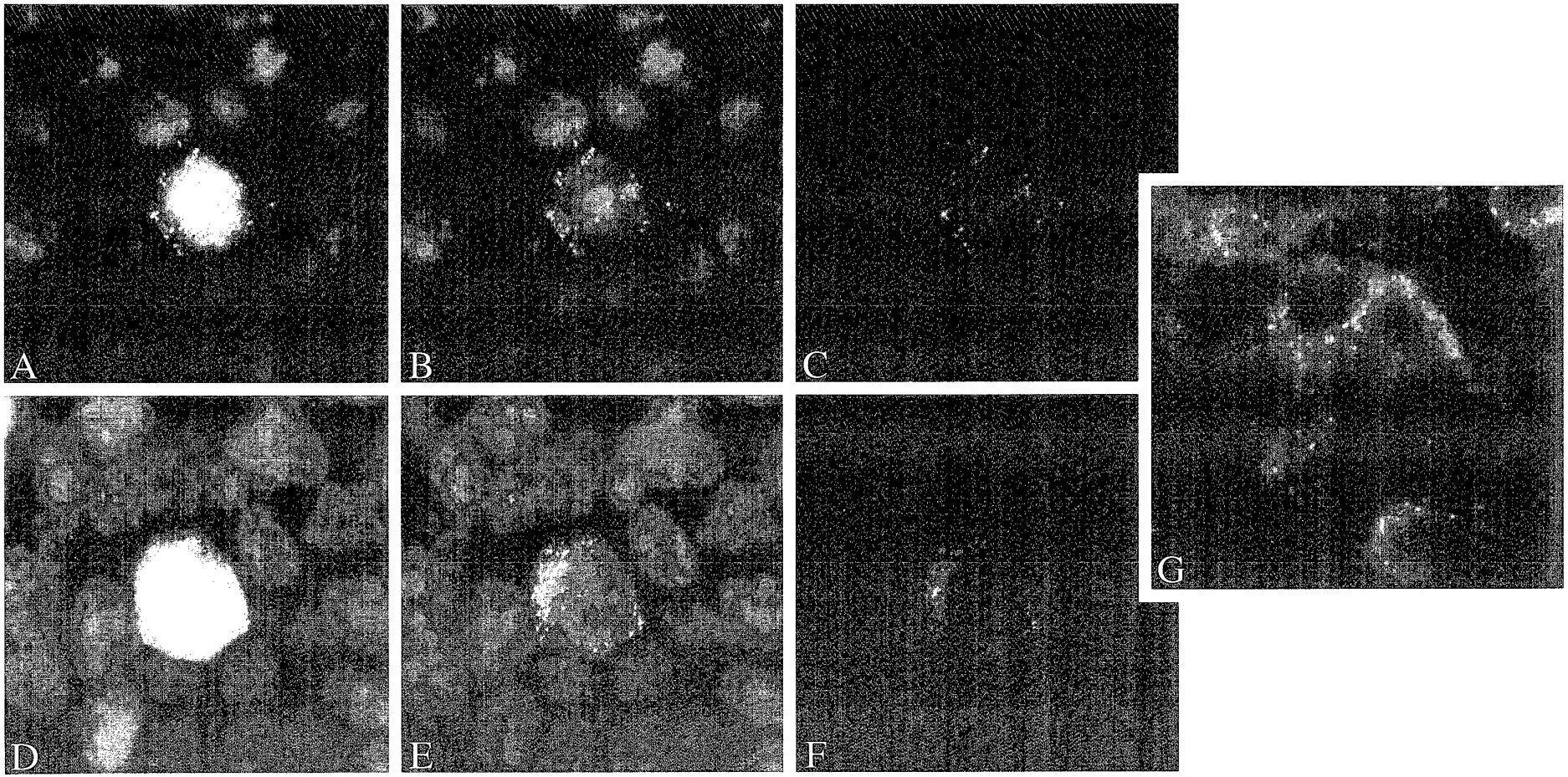

Figure 9. Effect of the FLAG tagged N-terminally truncated occludin construct on the localization of fascin 28 hours post transduction:

Filter grown CIT3 cells were treated with a multiplicity of infection (moi) of ~50pfu/cell of an E1/E3 deleted adenovirus expression vector encoding the occludin transgene and GFP. The filters were processed for immunofluorescence 28 hours following viral treatment. Filters were incubated with a mouse monoclonal antibody against fascin and then treated with a Cy3 (red) conjugated anti-mouse secondary antibody. Antibody treated filters were then stained with dapi nuclear dye (blue): A-F = 100x; A-C = same cells; D-F = same cells; A and D = fascin, dapi, and GFP; B and E = fascin and dapi; C and F = fascin. G = fascin staining in nontransduced cells.

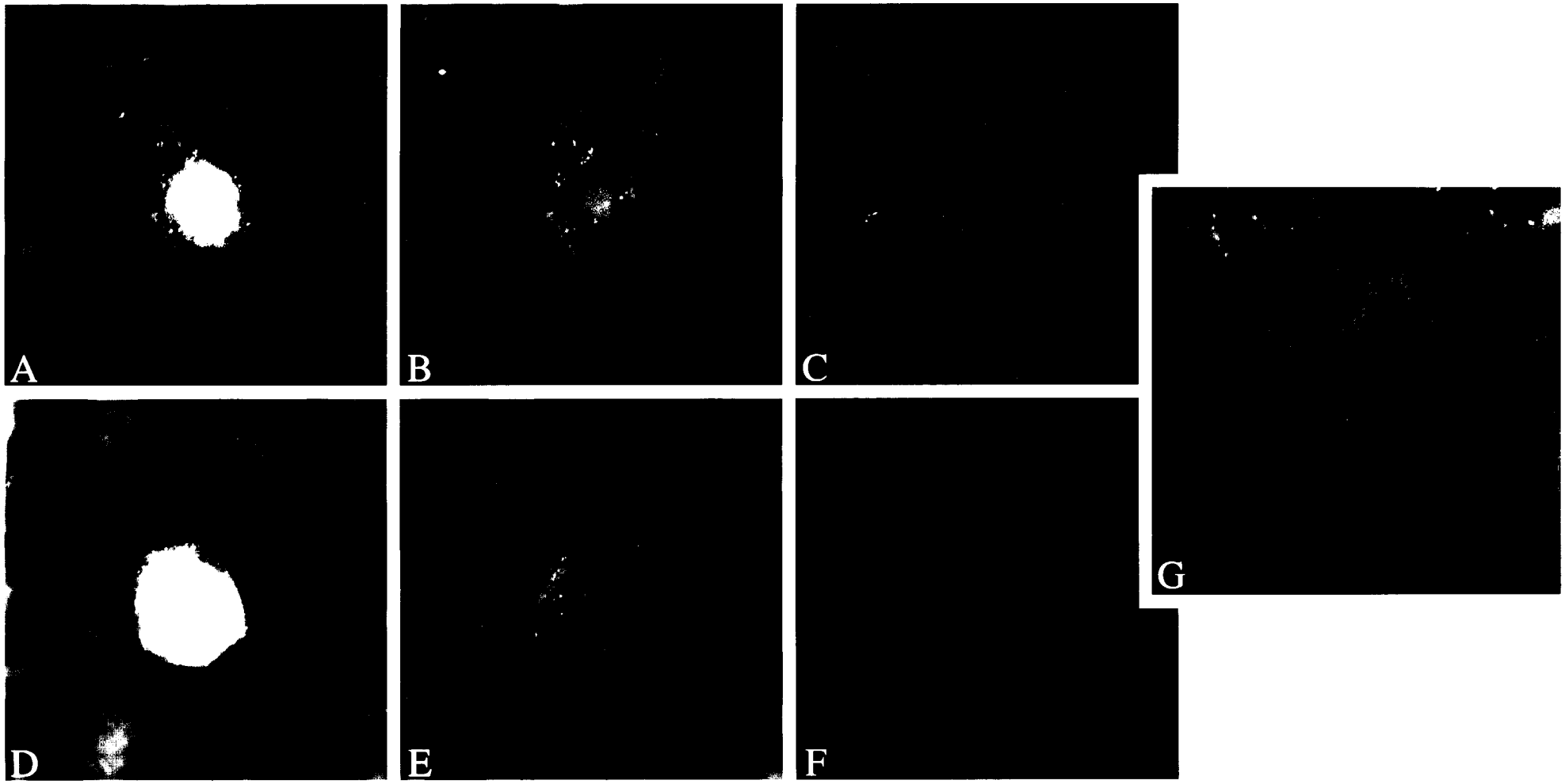

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Figure 10

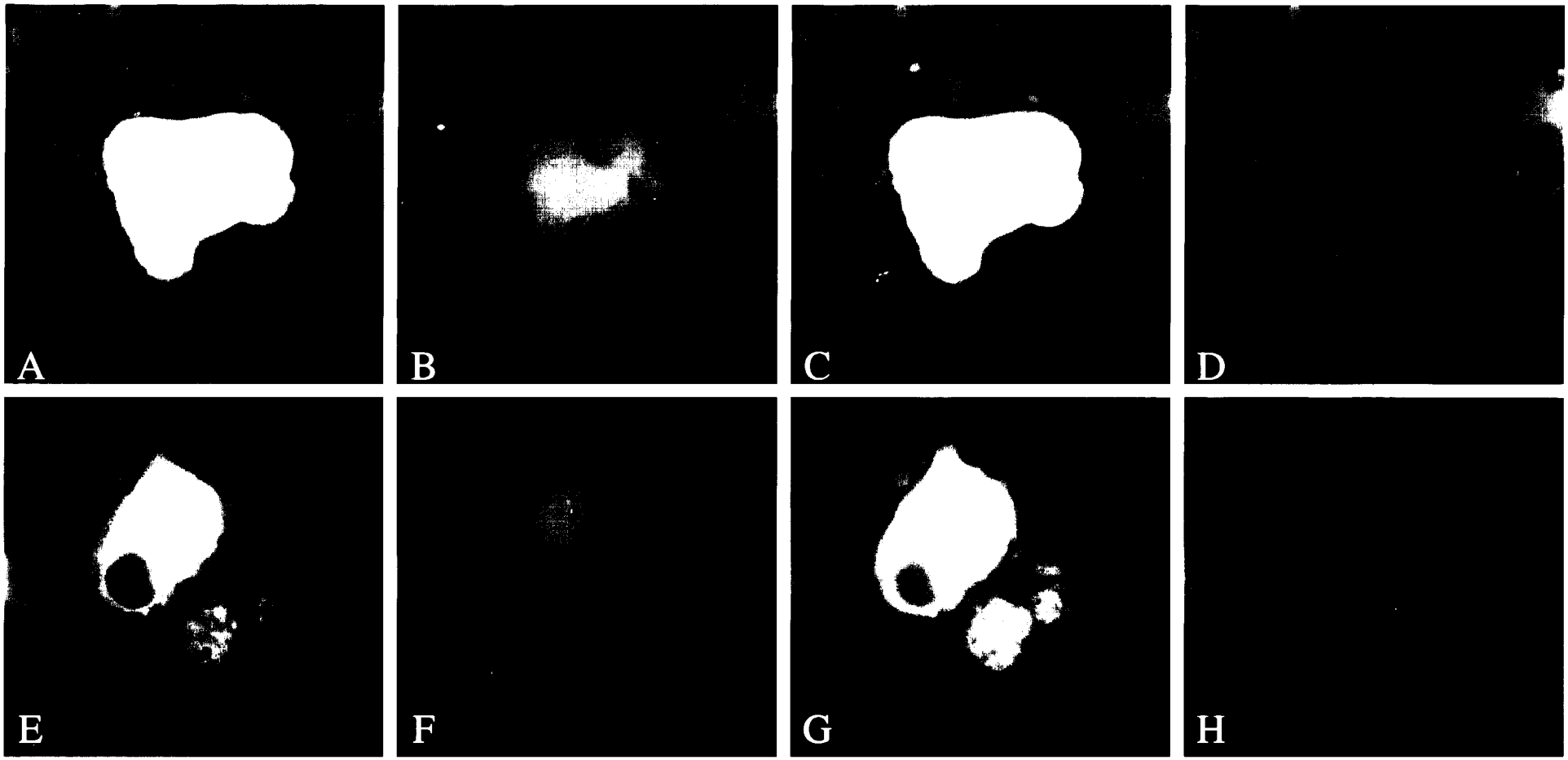

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Figure 11

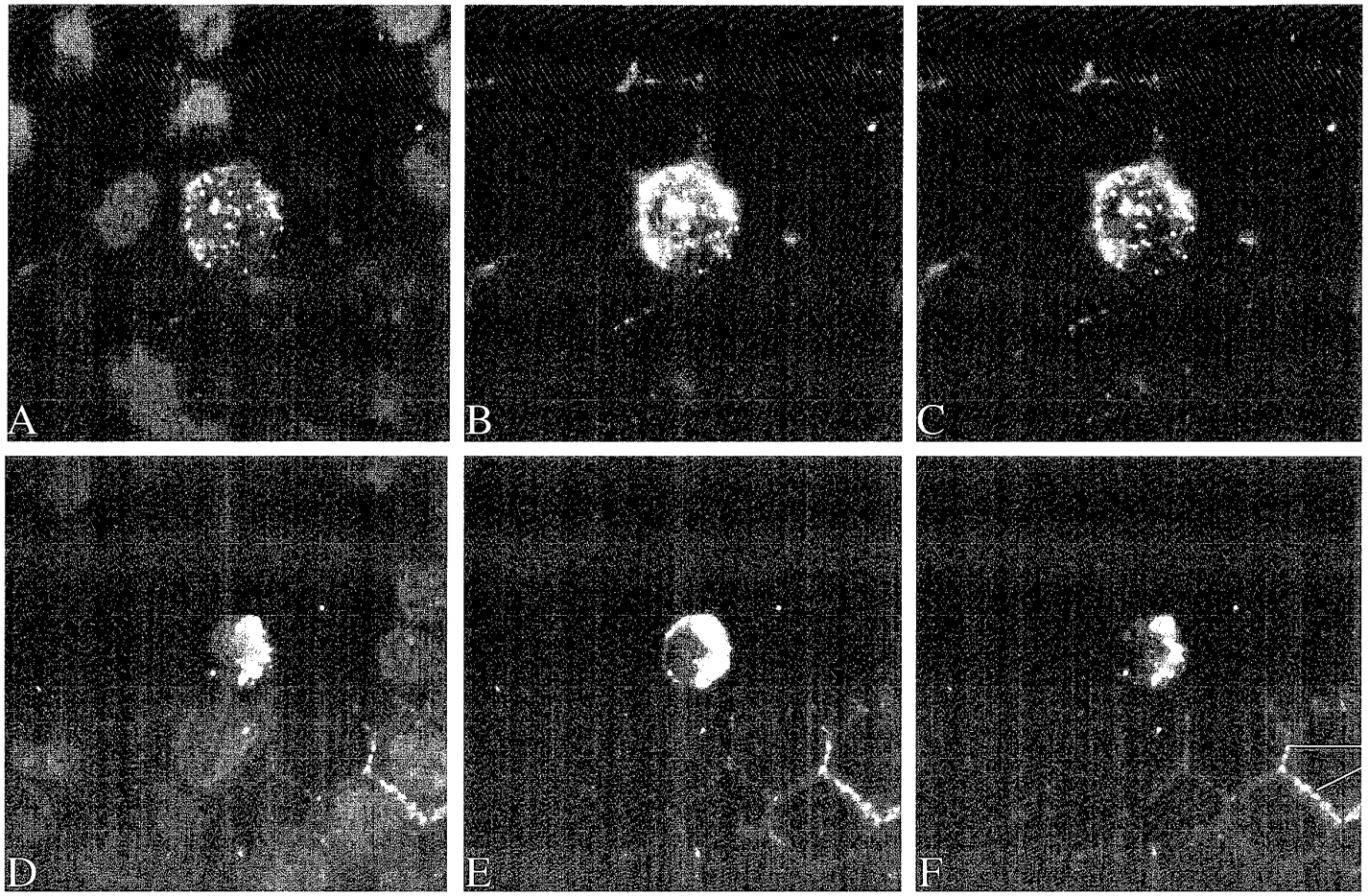
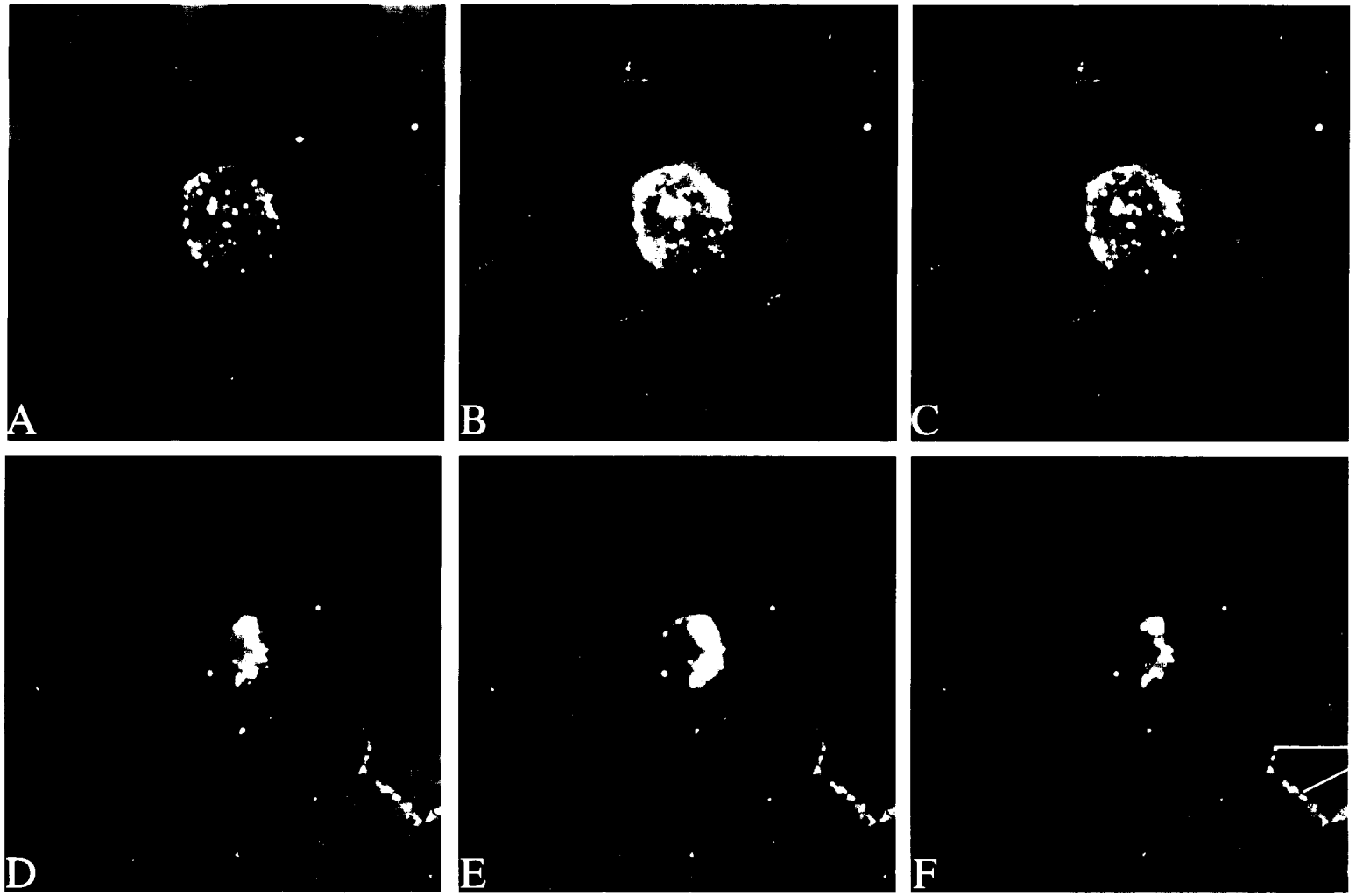


Figure 11. Effect of the FLAG tagged N-terminally truncated occludin construct on the localization of β-catenin:

Filter grown CIT3 cells were treated with a multiplicity of infection (moi) of ~50pfu/cell of an E1/E3 deleted adenovirus expression vector encoding the occludin transgene and GFP. The filters were processed for immunofluorescence 48 hours following viral treatment. Filters were incubated with a mouse monoclonal antibody against FLAG and a goat polyclonal antibody againstβ-catenin. Sections were then treated with Cy3 (red) conjugated anti-mouse and Cy5 (shown in green) conjugated anti-goat secondary antibodies. Antibody treated filters were then stained with dapi nuclear dye (blue): A-F = 100x; A-C = same cell; D-F = same cell; A and D = β-catenin and dapi; B and E = FLAG and β -catenin; C and F = β -catenin.



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